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Fundamental Procedures for Determining Ergosterol Content of Decaying Plant Material by Liquid Chromatography†

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Portions of published procedures for measurement of ergosterol content of decomposing plants were examined for their influence upon ergosterol yield. Common methods of treatment of plant samples prior to sterol extraction (e.g., oven drying, freezing, lyophilization) led to reduced recoveries of ergosterol (ca. 20 to 80%). The least destructive method was direct placement and storage in methanol. Photoconversion of ergosterol is not likely to cause losses during analysis, but losses are likely if there is insufficient mixing during neutral-lipid partitioning from base-hydrolysis reagents. Homogenization (two times for 2 min) and refluxing (2 h) in methanol were equally effective in extracting ergosterol. Direct extraction in base-hydrolysis reagents was less effective (by ca. 40%).

Ergosterol (24β-methylcholesta-5,7,*trans* 22-trien-3β-ol) has gained in popularity as a quantitative chemical index for fungal mass (e.g., references 10, 13, 17, and 27) since its introduction as such (8, 21, 22). The facts that the 5,7-double bonding of ergosterol permits sensitive assay by measurement of the A_{282} of UV light in neutral-lipid extracts and that this particular double bonding is rare among major sterols of vascular plants (but see references 14 and 24) and nonfungal saprotrophic microbes (26) make ergosterol a particularly useful fungal-index molecule. It may be that it will serve better in this regard than glucosamine (14; but see reference 15) or that it can be used in conjunction with glucosamine analysis to yield ratios of live/dead fungal mass (27; see also reference 28).

In developing the ergosterol-fungal method for ecological application, three independently working investigators presented quite different procedures for extraction and analysis. Seitz et al. (21, 22) extracted grain or fungal samples by homogenizing (2 min) in methanol; they assayed ergosterol by high-pressure liquid chromatography (HPLC). Lee et al. (7, 8) extracted leaves and stems by refluxing (96 h) in toluene-methanol; they assayed ergosterol as its acetate derivative by capillary gas chromatography. Griffiths et al. (4) extracted and saponified leaf samples in one step (with ethanol-KOH, 1 h) and assayed by HPLC. It is not known whether one procedure is more efficient than another in yielding ergosterol from natural substrates. This information is important if ergosterol is to be useful as a quantitative measure of fungal biomass, especially in view of the fact that molecules related to ergosterol are labile upon exposure to light, heat, and chemical reagents (25). We have compared various periods of methanol refluxing (with and without KOH) and various lengths of methanol homogenizing for four types of salt marsh plant materials, followed by HPLC assay. In addition, we have examined the effects of different types of sample storage, sample processing, and exposure of ergosterol to light to test the resistance of ergosterol to degradative and handling loss.

All plant materials used were collected from salt marshes of Sapelo Island (18). Standing dead leaves were collected for *Spartina alterniflora* Loisel., *Spartina patens* (Aiton) Muhl., and *Juncus roemerianus* Scheele, and standing dead stems (leaf sheaths removed; stem diameter, 4 to 5 mm) were collected for *S. alterniflora*. Leaves and stems were rinsed under running tap water, with rubbing by hand to remove adherent clay. For each leaf, a 10-cm portion, beginning 10 cm distal to the ligule, was cut into 1-cm lengths. Stems were cut into 0.5-cm lengths. Plant pieces were pooled and mixed, and five pieces of plant were allotted nonselectively to treatment replicates. After allotment, each piece of *S. alterniflora* was cut lengthwise to open stems and tightly curled leaves. During each experiment, 2 to 3 parallel replicate samples were used for determination of mean dry weight per replicate (drying at 100°C; range of mean replicate weights, 38.3 mg for *S. patens* to 75.1 mg for *S. alterniflora*).

HPLC-grade methanol, methylene chloride, isopropanol, and pentane were purchased from Fisher Scientific Co. Ergosterol was purchased from Fluka Chemical Corp., and ergocalciferol was purchased from Aldrich Chemical Co., Inc.

Extractions involving homogenization (10) were carried out in 15 ml of methanol in 50-ml centrifuge tubes in an ice bath (Polytron with PT20ST generator, 2.1×10^4 rpm; Brinkman Instruments, Inc.). The generator was rinsed (5 ml of methanol) into the sample. Subsequent centrifugations of homogenates were for 5 min at $875 \times g$. Supernatants were collected, and centrifugates were homogenized and centrifuged again. Primary and secondary supernatants were combined; final centrifugates were discarded.

Reflux extractions (no homogenization) were performed in glassware with Teflon-faced screw-thread connections (Wheaton Scientific) in 25 ml of methanol. Reflux flasks were submerged in a water bath held at 80°C. Hydrolysis of sterol esters was effected for both types of extraction by addition of an inert boiling chip and 5 ml of a 4% solution of KOH in 95% ethanol to each reflux flask and by refluxing for 30 min. With the exception of one experiment, solids were removed by centrifugation or with forceps before the addition of base.

Sterols were removed from the alcoholic base by partitioning into pentane after cooling to 25°C and by the addition of 10 ml of distilled water in 65-ml glass screw-cap tubes with Teflon-lined caps. A series of three pentane additions (10, 5, and 5 ml) were mixed by repeatedly inverting tightly sealed

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tubes; after each addition and mixing, the upper, pentane layer was withdrawn and pentane fractions from each sample were pooled. Pentane fractions were evaporated in open glass vials in a Temp-Blok (Scientific Products) set at 30°C under streams of nitrogen or air in a fume hood. The dried-down samples were redissolved in 1 to 3 ml of methanol, methylene chloride-methanol (1:1), or methylene chloride-isopropanol (99:1); vials were tightly sealed and sonicated for 5 min (Buehler Ultramet III Sonic Cleaner) to enhance the rate of dissolution (20). Of the three injection solvents, methanol gave the narrowest peaks for ergosterol; a slight shoulder was sometimes seen for methylene chloride-isopropanol. Use of methanol also eliminated injection-solvent peaks.

Redissolved samples were filtered through 0.45-μm-pore-size polytetrafluoroethylene membranes (Acrodisc 13CR; Gelman Sciences, Inc.) before injection into the following HPLC system: Dionex Analytical Pump APM-1 and pneumatic injection valve (50-μl sample loop) (Dionex Corp.); Pierce RP-18, 10-μm, 4.6-mm-inside-diameter, 22-cm column, with RP-18, 7-μm, 1.5-cm guard (Pierce Chemical Co.); Kratos Spectroflow 783 absorbance detector (Applied Biosystems); and Dionex 4290 integrator. At a flow rate of methanol eluant of 2 ml · min^{-1} (350 lb · in^{-2}) and a detector wavelength of 282 nm, ergosterol was detected approximately 3.6 min after injection. The detection limit for ergosterol for this assay system was ca. 1 μg · ml^{-1} of injection fluid. Solvent blanks taken through the extraction procedure did not contain detectable ergosterol.

Several experiments were conducted which compared variants of the general scheme presented above and determined effects of handling and storage upon ergosterol, as described below. An abridged flowchart of a suggested analytical procedure incorporating information shown below is given in Fig. 1.

Solutions of standard ergosterol (100 μg · ml^{-1}) in 10 ml of methylene chloride-methanol (1:1) were stored in scintillation vials in darkness for 0, 1, 7, and 14 days at −20, 4, and 25°C (number of replicates [n] = 3). There was no detectable loss of ergosterol with any treatment (analysis of variance, $P > 0.05$). The average difference from the zero-day mean was −0.5%. Vials of the same solution were also exposed to light under varied conditions, as presented in Table 1. Foil-wrapped controls showed no loss relative to controls kept in darkness at 4°C in the laboratory. Losses of ergosterol were not detected for samples exposed to fluorescent lamps, whereas exposures longer than 2 h to outdoor sunlight on a bright, sunny day did bring about losses (Table 1). Peaks corresponding to the retention time of standard ergocalciferol appeared in the dissolved samples exposed to outdoor sunlight at 40°C.

We tested three other portions of the extraction procedure for effects upon ergosterol standards: (i) extended refluxing in the alcoholic base, (ii) drying down of pentane solution with streams of nitrogen versus air, and (iii) extent of mixing of pentane with water and alcoholic base. Refluxing in base for up to 2 h did not cause loss of ergosterol. Nitrogen and air gave equivalent results (see reference 1,p. 483–484), indicating that one need not buy nitrogen gasfor this procedure (however, the flash point of pentane is <–40°C). However, inadequate mixing (10 inversions, repeated three times) gave only 56% recovery, as opposed to 100% recovery with 30 inversions three times (see reference 1, p. 484).

Types of preextraction storage of samples of *S. alterniflora* leaves were tested in two experiments. In one case, leaves were either dried to constant temperature at 50°C, frozen at −20°C, or lyophilized (freezing [to −30°C over ca. 30 min] and vacuum drying in one unit; Labconco Vac-Stop + Freeze Drier 8). These samples were extracted directly following treatment. In the second experiment, leaf samples were either lyophilized and stored over fresh desiccant in a

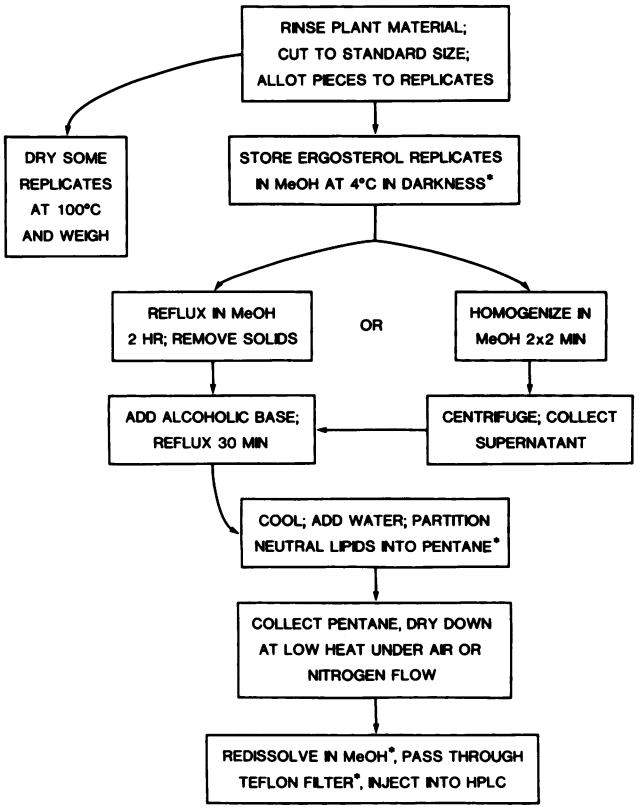

FIG. 1. Flowchart of the basic steps of a suggested procedure for analysis of ergosterol by HPLC. The procedure is derived from those presented in references 10, 21, and 22. *, Point at which samples can be stored without loss of ergosterol. MeOH, Methanol.

TABLE 1. Effect of light on ergosterol standards in tightly capped scintillation vials[a]

Light source and state of ergosterol	Light intensity[b]	Exposure		Difference from control (%)[c]
		Time (h)	Temp (°C)	
Fluorescent lamps, solution[d]	30	1	25	-1.02^{NS}
	160	1	25	$+0.01^{NS}$
Filtered sunlight, solution[e]	1,200	1	36	-1.80^{NS}
	1,100	2	32	$+1.18^{NS}$
Sunlight, solution[f]	1,682	2	23	-3.17^{NS}
	1,458	4	40	−28.62*
Sunlight, dry film[g]	1,725	2	36	−12.64*
	1,458	4	40	−81.03*

[a] Light intensity within vial reduced by 30% at center of base.
[b] Microeinsteins · $meter^{-2}$ · $second^{-1}$ photosynthetically available radiation (Li-Cor LI-188B integrating photometer), average for exposure period.
[c] Mean of 2 to 3 replicates. NS, Not significantly different from aluminum-foil-wrapped control; *, significant loss ($P < 0.05$).
[d] 10 cm or 1.5 m from lamps, 100 μg · ml^{-1}.
[e] Through a window pane, 100 μg · ml^{-1}.
[f] Outdoors, 40 μg · ml^{-1}.
[g] Outdoors, 200 μg dried down at the base of the vials.

TABLE 2. Relative yield of ergosterol from dead plant materials by either homogenization in methanol or refluxing in methanol

Plant material[b]	Relative yield[a] of ergosterol after:						
	Homogenization (min)[c]				Refluxing (h)		
	1	2	4	6	2	4	8
SA leaf	92	100	97				
SA leaf		77			100	85	97
JR leaf		100			87		
SP leaf		100			94		99
SA stem		100	69	91	90	89	

[a] Given as percentages of the highest mean value for each row; mean of 2 to 3 replicates; no significant differences detected within any row (analysis of variance, $P > 0.05$). Yields are comparable within rows.
[b] SA, *Spartina alterniflora*; SP, *Spartina patens*; JR, *Juncus roemerianus*.
[c] Each homogenization was performed twice for each sample.

partial vacuum for 1 week at 25°C, lyophilized and stored at 50% relative humidity at 25°C for 1 week, or placed directly into methanol and stored at 4°C for 1 week. When compared to freeze-thawing and mild oven drying, lyophilization gave the best yield of ergosterol. As mean percentages of the yield after lyophilization, oven drying gave 23% and freeze-thawing gave 86% (freezing versus lyophilization, $0.10 > P > 0.05$). When compared with direct placement in methanol, lyophilization provided 20% less yield of ergosterol, regardless of type of subsequent storage ($P < 0.05$ for pooled lyophilization treatments).

During the storage experiments, we compared recoveries for standard ergosterol, either adding 50 μg of standard to methanol alone prior to refluxing or adding it as a spike to extra sample replicates. Recoveries for the two methods of addition were not statistically different (range, 95 to 107%; $n = 2$ to 3).

Times of homogenization were tested and compared with various periods of methanol refluxing of unhomogenized samples in several experiments (Table 2). Two minutes of homogenization (two times) in methanol was enough to give a maximum yield of ergosterol for the two types of dead plant material tested. Refluxing in methanol for 2 h gave yields equivalent to homogenization. Longer refluxing did not change yields. Mean values for ergosterol content ($\mu g \cdot g^{-1}$ dry weight) in the standing dead material were as follows: leaves of *S. alterniflora*, 392; leaves of *S. patens*, 263; leaves of *J. roemerianus*, 202; stems of *S. alterniflora*, 312.

We tried moving directly to the alcoholic-base-hydrolysis refluxing (30 min) without homogenizing or centrifuging samples of *S. alterniflora* leaves. This procedure yielded an average of 37% less ergosterol ($P < 0.05$; $n = 3$) than the 2× 2-min homogenization-centrifugation procedure followed by alcoholic-base refluxing of supernatant. We also tested three different times (15, 30, and 60 min) of alcoholic-base refluxing with *S. alterniflora* leaf samples, following 2× 2-min homogenization; there was no significant difference among mean yields of ergosterol ($P > 0.05$; $n = 3$), which were within 5% of one another.

The literature suggests that ergosterol should not be exposed to light during extraction (e.g., reference 23). This probably stems from the fact that ergosterol (alias provitamin D_2) forms vitamin D_2 (ergocalciferol) and two different sterols, by way of an intermediate, upon exposure to UV irradiation (6, 25). However, 2 h of exposure to direct, simulated, equatorial solar UV irradiation is required for photoconversion of 50% of provitamin D_3 (7-dehydrocholesterol) (6), and our results indicate that without severe exposures to very bright light, ergosterol is even more resistant to photoconversion losses (Table 1). This is consistent with the results of Nout et al. (15); 20-h exposure of ethanol solution of ergosterol (50 $\mu g \cdot ml^{-1}$) to diffuse daylight led to 1.6% loss. Therefore, one need not be overzealous in protecting extracted ergosterol samples from light in the laboratory. Also, extracted ergosterol is resistant to losses in dark storage for at least several days, even at room temperature; thus, as long as evaporation is prevented, sample-processing schedules can include lengthy interruptions before injection into the HPLC system.

Samples intended for ergosterol analysis have been treated for preextraction storage by oven drying (e.g., references 9 and 16), freezing (e.g., references 11 and 17), and lyophilization (e.g., references 4 and 13). Our findings indicate that all of these treatments, especially oven drying, have the potential for leading to lower recovery of ergosterol from plant samples. Since extracted ergosterol withstands boiling in methanol (65°C) for hours, it is likely that the oven-drying losses, and probably also the freeze-thawing and lyophilization losses, occur as the living fungal mass within the plant samples is killed. Our lyophilization yields might have been higher if the samples had been instantly frozen, preventing autolysis. The optimal storage procedure appears to be to place the samples directly into the extractant (methanol).

Lipids, including sterols, are traditionally extracted from natural tissues by lengthy extraction or brief homogenization in nonpolar solvents, including methanol (2, 19). Each of these methods has been used as part of ergosterol analytical procedures (8, 22). Our direct comparisons of the two types of procedures (Table 2) show that they can be used interchangeably and that two 2-min cycles of homogenization or 2 h of methanol refluxing provides equivalent, maximal yields. This was true even for highly lignified plant materials (e.g., stems of *S. alterniflora*; see reference 5). Direct extraction in alcoholic base (saponification reagents), in contrast to the findings of Nes et al. (12), gave reduced yields of ergosterol; this may have been a result of sterol adherence to complex polysaccharide molecules at the surfaces of plant particles (3).

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